A STUDY OF THE THERAPEUTIC MECHANISM OF ANTI-PNEUMOCOCCIC SERUM ON THE EXPERIMENTAL DERMAL PNEUMOCOCCUS INFECTION IN RABBITS

I. The Presence in Antipneumococcic Serum of a Non-Antibacterial Therapeutic Factor*

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Despite the numerous clinical and experimental investigations on antipneumococcic serum, its exact rôle in pneumococcus infection is still relatively obscure. The purpose of this communication is to report experiments which show that antipneumococcic serum contains an important therapeutic factor, which is not antibacterial in character.

The current opinion is that the protective antibody in the serum is antibacterial. In 1915, Bull (1), in studying the effect of serum on pneumococcus septicemia in rabbits, suggested that the protective antibody acts *in vivo* by virtue of its agglutinating power, and that the survival or death of the animal depends upon the destruction of the agglutinated organisms by the phagocytic cells of the blood and tissues. Cole (2), Dochez and Avery (3), and others showed that in pneumococcus-infected exudates and sera as well as during the growth of the organisms *in vitro*, there is present a soluble substance, which can combine with the immune bodies of the serum, and thus detract from its action against the organisms. The later studies of Heidelberger and Avery (4), and others on the chemical constitution of pneumococcus protoplasm had established this soluble specific substance as the chief capsular constituent, a carbohydrate fraction of the organism responsible for its type specificity and virulence. The subsequent work of Felton and Bailey (5) indicated that this purified carbohydrate soluble specific substance was capable of neutralizing the protective antibody. Thus it appeared that the func-

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tion of antipneumococcic serum is to combine with the soluble specific substance present either free in the circulation or on the intact organism. This concept of the mechanism, however, fails to explain all the known facts.

For instance, further investigations (6) showed that in addition to the antibody neutralizable by the soluble specific substance (SSS), antipneumococcic serum contains type-specific antibody which is not thus neutralized. This fact was established by demonstrating first, that a certain amount of type-specific protective antibody remains in antipneumococcic serum after complete precipitation of the anti-SSS precipitins, and second, that this residual type-specific protective antibody is neutralized neither by additional SSS nor by absorption with heterologous pneumococci; it is, however, definitely absorbed with the homologous pneumococci. Recently, Ward (7) questioned the validity of this assumption first because he felt that it appeared improbable that the protective action of the serum should be due to several antibodies, and secondly because he was able to show that a Type III serum apparently completely precipitated with Type III SSS, still had a certain amount of antibody, which must have escaped precipitation, and was capable of specifically neutralizing the *in vitro*, specific antiphagocytic effect of small amounts of SSS. With regard to the first contention, it is not at all improbable that the antibacterial effect of a serum may depend on several antibodies reacting with different antigenic components of the organism. Actually the work of Enders (8), and more recently that of Wadsworth and Brown (9) indicate that a type-specific substance other than the so called SSS does exist in Pneumococcus Type I. Whether or not the same is true in the case of Pneumococcus Type III is still unknown and, therefore, the comparison is not warranted. With reference to Ward's repetition of the experiments, referred to above (6), it must be stated that he failed to add more SSS to the supernatant liquid of his apparently completely precipitated serum, before testing its neutralization of the antiphagocytic effect of small amounts of SSS. What the results would have been then is, of course, impossible to predict, although theoretically, the solutions, in which the residual type-specific mouse protective antibody was demonstrated (6), because they contained a large excess of SSS would rather be expected to be antiphagocytic *in vitro*. Nevertheless, it is interesting to note that Ward's other experiments suggested that the anti-SSS action is not the only function of the serum.

However, whether there be one, two, or more type-specific antibodies, their action is nevertheless chiefly antibacterial, since they can be specifically absorbed with the intact organisms, and their *in vivo* rôle may still come under the agglutinin effect as conceived by Bull (1). Yet the nature of the factors causing the toxemia and death of pneumococcus-infected animals remains obscure. Certainly the mere presence of the organisms as innumerable microscopic particles cannot account for this effect. Theoretically, therefore, it has been assumed for a long time that the breakdown of the organisms *in vivo* gives rise to toxic substances. The soluble specific substance which neutralizes some of the antibacterial antibody

is apparently *per se* non-toxic to mice, rabbits, or human beings.[1] The studies of Rosenow (10), Cole (11), and others on pneumococcus autolysates showed that while these produce anaphylactic-like reactions in animals, they could not be correlated with the signs of toxemia in pneumococcus infection. In the past few years, Parker (12) has described certain toxic substances obtained by anaerobic autolysis of concentrated pneumococcus suspensions. The toxic principles in these autolysates are capable of producing necrosis when injected intracutaneously and death associated with hemorrhagic pulmonary lesions when injected intratracheally into guinea pigs. These toxins are species-specific and can be neutralized by heterologous anti-autolysate serum. Parker and McCoy (13) reported the production of potent antitoxic serum in horses. However, it appeared that if this toxin prepared *in vitro* by Parker played any part in the *in vivo* process, that the antitoxic serum should act not only on a lesion produced by the artificial toxin, but should exert a definite effect on the course or outcome of the natural infection as well. This problem was investigated using the dermal pneumococcic lesion of rabbits reported by Zinsser (14) and later studied in detail by Goodner (15), as it seemed to be the most suitable, available type of experimental pneumococcus infection, particularly since its course and pathology (Rhoads and Goodner (16)) resemble so greatly that of pneumococcus pneumonia in man. It appeared from this study (17) that the antitoxic serum of Parker, when used by itself or in association with a so called subeffective dose of antibacterial serum, had no apparent effect on the progress of the pneumococcus infection in rabbits. For this reason it would seem that the anaerobically isolated toxin is probably merely a product of *in vitro* autolysis and not actually formed in the course of pneumococcus infection.

The late Dr. E. J. Banzhaf, similarly interested in producing a serum that might perhaps be antitoxic as well as antibacterial, injected horses intramuscularly with pneumococcus broth culture filtrates as well as with the sedimented organisms intravenously. He assumed that in case pneumococcus broth cultures should contain a toxin, which, however, is not readily demonstrable on account of a probable rapid transformation into a toxoid stage, it might, nevertheless, be possible to prepare an antitoxin if they were used for immunization. Banzhaf's preliminary laboratory tests with sera thus produced indicated that some rabbits could be protected with quantities, in which the number of mouse protective units was surprisingly low. He subsequently very kindly submitted to me several samples of this serum for further study, the results of which are included in this report.

The following experiments deal with the demonstration of the presence or absence of therapeutic agents in antipneumococcic serum which

[1] The author injected four volunteers intramuscularly with as much as 5 mg. of Type I SSS (the equivalent of 200 to 500 cc. of culture) without any harmful effects in three; one man, who had demonstrable agglutinins for Type I pneumococcus in his blood, and was probably hypersensitive, had a rise of temperature to 102.5°F. for 18 hours and marked local pain for 3 days.

are not antibacterial in character. For this study, the dermal pneumococcic lesion of rabbits appeared to be the most suitable, available experimental pneumococcus infection.

EXPERIMENTAL

Pneumococcus Culture.—A 5 per cent blood broth culture of a fully virulent (*i.e.* one to five diplococci, as determined by colony count in poured plates, killed mice in 3 days or less) Type I Pneumococcus was used in all the experiments. The culture was maintained in this state of virulence and supplied for each experiment by Miss Georgia Cooper of the New York City Department of Health Research Laboratories, to whom I am greatly indebted.

The Dermal Pneumococcic Lesion in the Rabbit.—Adult rabbits weighing 1500 to 2000 gm. were used. The abdominal hair was removed with electric clippers the day before injection. 1 cc. of an 18 hour blood broth culture was diluted with 1 or 2 cc. of broth to match a turbidity standard, so as to yield approximately 200,000,000 diplococci per cc. The further dilutions were also made with broth, using a fresh pipette for each dilution. 0.1 cc. of a 1–100 dilution of the standardized culture was injected intracutaneously into each rabbit. The number of organisms injected were checked each time by a colony count in poured blood plates.

Rectal temperatures were taken before the injection and daily thereafter throughout the duration of the experiment. Blood cultures from the marginal ear vein were taken daily from each rabbit. The lesions were measured every day noting the size, edema, erythema, hemorrhage, and necrosis. Lesion cultures were not done routinely because only a positive culture was considered significant, and because the elimination of the necessary manipulation was deemed more important as regards these experiments than the additional data that might have been obtained. All rabbits were thus observed for at least 10 days. Each dead rabbit was autopsied and cultures were taken from the heart's blood and from the brain.

Comparison of the Therapeutic Effect of Standard Serum and That Prepared by Intravenous Injection of Organisms and Intramuscular Injection of Broth Culture Filtrate

The purpose of this experiment was to determine, in a preliminary way, whether or not the antiserum prepared by immunization with organisms and broth culture filtrates was therapeutically more effective in rabbits than the standard serum, prepared by intravenous injections of sedimented organisms only.

In this, as well as in the subsequent experiments, the serum was administered intravenously 6 to 7 hours after the intracutaneous injection of the culture

rather than at 24 hours, as Goodner had done in his studies, because it appeared more suitable for comparative purposes. The virulence of the culture was such that, with the dose used, of forty untreated rabbits in various experiments, none survived; furthermore at 24 hours the disease is frequently very far advanced, some of the rabbits dying within 48 or 72 hours; at 6 hours, however, the lesion is very slight and the blood culture is positive, so that the effect of the serum can be noted not only on the outcome of the disease but on the progress and development of the lesion as well. It is important to note here that protective doses administered at 6 hours do not necessarily influence the full development of the lesion.

TABLE I

Therapeutic Effect of Standard Antipneumococcic Serum and Serum Prepared by Injections of Organisms and Broth Culture Filtrates

Therapy	Dose		Hrs. after culture	No. of rabbits used	No. survived
	cc.	*m.p.u.**			
None	—	—	—	4	0
Standard Serum 8 Type I	0.38	300	7	4	1
Serum 2321 Type I	5.0	300	7	2	2
	5.0	300	24	2	2
	0.5	30	7	1	0
	0.5	30	24	1	1

* *M.p.u.*, mouse protective units. The mouse protective unit is ten times the smallest amount of antiserum, which protects a majority of mice for 96 hours, when it is injected intraperitoneally simultaneously with 100,000 fatal doses of a fully virulent culture.

From previous experiments it was known that 300 mouse protective units (m.p.u.) of standard Serum 8 were insufficient for the protection of a majority of rabbits receiving this dose at 6 hours after infection. Serum 2321, produced by intravenous injection of organisms and intramuscular injection of broth culture filtrate, was obtained from a horse that had been under immunization for a relatively short time and its mouse protective potency was rather low. In this experiment four rabbits were untreated; four received 300 units of Serum 8 at 7 hours; 2 rabbits, 300 units of Serum 2321 at 7 hours, and 2 rabbits the same dose at 24 hours; one rabbit, 30 units of Serum 2321 at 7 hours, and one the same dose at 24 hours.

The results are presented in Table I, and show that all the rabbits treated with 300 m.p.u. of Serum 2321 survived, whereas of four rab-

bits treated with 300 m.p.u. of Serum 8, three died. It thus appeared that an equal or similar dose (as regards mouse protective units—the unit which is used clinically as the criterion of dosage) of two different sera was in one instance fully protective for rabbits and in the other practically ineffective. In addition to the variations in the immunization procedures that were used in the preparation of the two sera, there was still another difference: standard Serum 8 contained the 300 m.p.u. in 0.38 cc., whereas Serum 2321 contained the same number of units in 5 cc. The question was, therefore, whether the greater effectiveness of Serum 2321 was due to the added intramuscular injections of broth culture filtrate employed in its preparation, or to a factor present in both Serum 2321 and Serum 8, more of which was associated with the 300 m.p.u. of the former on account of the larger volume of the dose. In either case, however, this factor would have to be of a nature different from the agent which is determined by the present method of mouse protection. By absorbing an antipneumococcic serum with the homologous organism it is possible to remove almost all (about 99.5 per cent) of its mouse protective capacity; it was, therefore, important to determine whether the supernatant liquid of a serum thus absorbed could exert any therapeutic effect in rabbits.

The Non-Antibacterial Therapeutic Factor in Serum Prepared by Injection of Organisms Intravenously and Broth Culture Filtrate Intramuscularly

In the work done on the rôle of anaerobic toxins in the dermal pneumococcic lesion of the rabbit (17), as well as in the previous experiment it was apparent that for every serum there is a certain subeffective dose. Of a group of rabbits treated with this subeffective dose there is at first a sterilization of the blood stream in all; in less than half the blood is reinfected whereas in the others it remains sterile; the dermal lesion is not markedly influenced; about 60 to 75 per cent die. Approximately half of these rabbits die without reinfection of the blood stream and with a sterile postmortem heart's blood culture, but usually with a progressively marked lesion. (Routine postmortem brain cultures, which were done on all the rabbits reported in these experiments, showed that, in the absence of a continued and persistent bacteremia, localization of the infection in the central nervous system was rela-

tively extremely rare.) It appeared, therefore, that in these cases death is produced by the absorption of toxins from the local lesion.

Thus, it was essential to determine whether a subeffective dose of serum could be rendered effective by the addition of that fraction of the serum which remains after complete absorption with the homologous organisms.

TABLE II

The Non-Antibacterial Therapeutic Factor in Serum Prepared by Injection of Organisms and Broth Culture Filtrates

Therapy	Dose		Hrs. after culture	No. of rabbits used	No. survived
	cc.	*m.p.u.*			
Preparation 4	0.2	50–100	6	9	4
Preparation 4	0.2	50–100	6		
+				3	1
Absorbed Preparation 4	0.4	0.4–2.0	24		
Preparation 4	0.4	100–200	6	5	2
Preparation 4	0.4	100–200	6		
+				5	5
Absorbed Preparation 4	0.6	0.6–3.0	24		
Preparation 4	0.4	100–200	6		
+					
Absorbed Preparation 4	0.25	0.25–1.25	24	5	5
	0.25	0.25–1.25	48		
	0.25	0.25–1.25	72		
Standard Serum 624 Type I	0.3	300	6	4	1
Serum 624	0.3	300	6		
+				4	4
Absorbed Preparation 4	0.5	0.5–2.5	6		
	0.5	0.5–2.5	24		

The serum available for this experiment was a concentrated preparation (Preparation 4) containing the total water-soluble and water-insoluble globulin (precipitated at 30 to 50 per cent saturation with $(NH_4)_2SO_4$) of Serum 2321 used in the previous experiment. This preparation contained 250 to 500 m.p.u. per cc. Preparation 4 was absorbed with heat-killed, sedimented pneumococci until no further agglutination occurred; when this stage was reached more organisms were

added, and the mixture incubated at 37°C. for 2 hours and kept in the refrigerator overnight. After sedimenting the unagglutinated organisms by thorough centrifugation, the supernatant liquid was poured off and called absorbed Preparation 4. A mouse protection test[2] performed with absorbed Preparation 4 showed that it contained 1 to 5 units, indicating an absorption of about 99.5 per cent of the original mouse protective capacity. In a preliminary experiment a subeffective dose of Preparation 4 was determined, then one group of rabbits was injected with a subeffective dose of Preparation 4 at 6 hours, and other groups received, in addition to the subeffective dose at 6 hours, injections of absorbed Preparation 4 at 24 hours, 48 hours, and 72 hours as shown in Table II.

The survival rate of the rabbits indicates that 0.2 and 0.4 cc. of Preparation 4 constitute subeffective doses. It is also clearly apparent that the additional injection of 0.6 cc. of absorbed Preparation 4 at 24 hours or of 0.75 cc. divided in three equal doses at 24 hour intervals rendered the subeffective dose therapeutically effective, all the rabbits thus treated having survived. As confirmatory evidence, it was necessary to determine whether absorbed Preparation 4 would have a similar effect when added to a subeffective dose of standard antibacterial serum; *i.e.*, one prepared by immunization with organisms only. The results shown in Table II again indicate that Preparation 4, after complete absorption with the homologous organisms, contains a substance which exerts a definite therapeutic effect on pneumococcus infection in rabbits. For convenience, this therapeutic factor was called non-antibacterial to indicate merely that it was not absorbed or neutralized by the intact homologous organisms, and not to define its mode of action. If this non-antibacterial factor is of the nature of an antibody or antitoxin, it apparently must be produced in response to some soluble toxic substance, which, however, is not an antigenic constituent of the intact organism.

Can Pneumococcus Broth Culture Filtrate Neutralize the Non-Antibacterial Factor?

Since broth culture filtrate was the additional immunizing agent used in the preparation of the serum, which was shown to contain the non-antibacterial therapeutic factor, it was important to determine

[2] The mouse protection tests reported in these experiments were performed in the laboratory of Miss Georgia Cooper of the New York City Health Department Research Laboratories, to whom the author again wishes to express his gratitude.

whether or not it would be capable of neutralizing this factor. It seemed that no causal relationship between the use of broth culture filtrate for immunization and the presence of the non-antibacterial factor in the serum could be established, unless such neutralization were demonstrable.

1000 cc. of a 24 hour Pneumococcus Type I broth culture were passed through a large Seitz filter. The filter was then concentrated by a method commonly used

TABLE III

Effect of Broth Culture Filtrate on Non-Antibacterial Factor

Therapy	Rabbit No.	Result	Remarks
0.3 cc. Serum 624 (300 units)	1	S	
+	2	S	
1.5 cc. absorbed Preparation 4	3	S	
	4	D_6*	Marked lesion; bacteremia controlled; postmortem heart and brain cultures sterile
0.3 cc. Serum 624 (300 units)	5	S	
+	6	D_4	Bacteremia uncontrolled
Mixture containing 1.5 cc. absorbed Preparation 4 and 3.0 cc. alcoholic pneumococcus broth concentrate	7	S	
	8	S	
0.3 cc. Serum 624 (300 units) only	9	S	
	10	D_6	Marked lesion; postmortem heart and brain cultures sterile
	11	D_3	" "
	12	D_9	" "
	13	D_3	Marked lesion; bacteremia uncontrolled

* S = survived. D_6 = died 6 days after injection.

in obtaining diphtheria toxin from broth cultures. The filtrate was acidified with glacial acetic acid and precipitated with 60 per cent alcohol; after remaining in the refrigerator overnight, the precipitate was filtered through paper. It was then pressed nearly dry between pieces of filter paper and dissolved in 50 cc. of 0.9 per cent NaCl; *i.e.*, in 1/20 the original volume. 2 cc. of this concentrate injected intravenously into two normal rabbits produced no symptoms. To one part of Preparation 4, which previously had been completely absorbed with heat-killed,

washed Type I pneumococci, two parts of the pneumococcus broth concentrate were added. The mixture was incubated in the water bath at 37°C. for 2 hours and kept in the refrigerator overnight. A small amount of precipitate had formed; this was centrifuged and the clear supernatant liquid was used for injection into rabbits. Four rabbits received a subeffective dose of standard Serum 624 together with 1.5 cc. of absorbed Preparation 4; four other rabbits—Serum 624 and a mixture of 1.5 cc. absorbed Preparation 4 treated with 3 cc. of the pneumococcus broth concentrate; five rabbits were injected with the subeffective dose of Serum 624 only, for control. All serum was given intravenously 6 hours after the intracutaneous injection of the organisms.

The results shown in Table III indicate that the concentrated pneumococcus broth culture filtrate failed to neutralize the therapeutic effect of absorbed Preparation 4. It thus appeared that if the broth culture filtrate does not neutralize the non-antibacterial therapeutic factor, it probably also does not contain its antigen, and that, therefore, serum prepared by the standard method of intravenous injection of organisms only, should similarly contain this factor.

Presence of the Non-Antibacterial Therapeutic Factor in Serum Prepared by the Intravenous Injection of Organisms Only

The purpose of this experiment was to determine first, whether or not the non-antibacterial therapeutic factor can be demonstrated in serum prepared by the intravenous injection of organisms only, and second, whether it is type-, species-, or non-specific. For control, normal horse serum, and Parker's so called antipneumotoxin, prepared by immunizing horses with the toxic, anaerobic, pneumococcus autolysate, were used.

The Type I sera were absorbed with Type I, heat-killed, washed pneumococcus suspensions; the heterologous serum, normal horse serum, and Parker's antipneumotoxin were not absorbed with any organisms, because a test showed that they contained less than 1 mouse protective unit per cc. for Type I pneumococcus. Four Type I monovalent sera, one Type II monovalent serum, and one normal horse serum are included in the tabulation of the results. The tests were carried out at different times on several different groups of rabbits. To determine whether the non-antibacterial factor could be demonstrated in a serum, other than antipneumococcic, one group of rabbits was tested with a scarlatinal, antibacterial, and antitoxic serum obtained from a horse that at no time had received any injections of pneumococci. The dose varied from 2 to 5 cc. for each serum tested. The results of all the tests are summarized in Table IV.

Of the forty rabbits that were untreated in the various experiments none survived. The striking contrast between the survival rate (29.0 per cent) of those receiving the subeffective dose of Type I serum only with that (81.4 per cent) of the rabbits receiving the additional injection of pneumococcus-absorbed Type I serum, indicates very definitely the presence of a similar non-antibacterial, therapeutic factor in serum prepared by the intravenous injection of organisms only. The results with the Type II serum are almost equally striking, and suggest that this non-antibacterial factor is not type-specific. The apparent absence of the factor in the antipneumotoxin is of

TABLE IV

The Non-Antibacterial Therapeutic Factor in Standard Antipneumococcic Serum

Serum therapy	No. of rabbits used	No. survived	Survival
			per cent
None	40	0	0
Subeffective dose of Type I antiserum	31	9	29.0
Subeffective dose of Type I antiserum + absorbed Type I serum	43	35	81.4
Subeffective dose of Type I serum + heterologous serum Type II	17	12	70.6
Subeffective dose of Type I serum + Parker's antipneumotoxin	22	6	27.3
Subeffective dose of Type I serum + normal horse serum	10	4	40.0
Subeffective dose of Type I serum + scarlatinal antibacterial and antitoxic serum	10	3	30.0

interest with regard to its mode of origin. The survival rates of the rabbits which received the normal horse serum and the scarlatinal, antibacterial and antitoxic serum, considering the smaller number of animals, are well within the range of survival of those treated with the subeffective dose only, the results indicating that this therapeutic factor probably is not non-specific. Considering the fact that standard antipneumococcic sera contain, in addition to the antibacterial antibody, other therapeutic factors which apparently are non-antibacterial, one can understand more readily how equal doses, as regards mouse protective unit content, may vary in therapeutic efficiency.

TABLE V

Therapeutic Effect of Non-Antibacterial Factor per Se and in Conjunction with a Subeffective Dose of Serum

Therapy	Experiment No.	Rabbit No.	Dermal lesion	Bacteremia	Result	Postmortem cultures	
						Heart's blood colonies per cc.	Brain
Absorbed Preparation 4, dose 2 cc. contained 2–10 m.p.u.	(*a*)	1	Very slight	Controlled	S		
		2	" "	Uncontrolled	D_4	1000	Sterile
		3	Slight	Controlled	S		
		4	"	"	S		
	(*b*)	5	Very slight	Controlled	D_2?*	Sterile	"
		6	Marked	"	D_7	"	"
		7	Moderate	"	D_{11}	"	"
		8	None	Marked; uncontrolled	D_2	Innumerable	Positive
		9	Slight	Controlled	S		
	(*c*)	10	Very slight	Controlled	S		
		11	" "	Uncontrolled	D_6	Innumerable	Innumerable
		12	" "	Controlled	S		
		13	Moderate	Uncontrolled	D_7	Innumerable	Positive
		14	"	Controlled	S		
		15	None	Uncontrolled	D_3†	Innumerable	Sterile
		16	Marked	Controlled	S		
Absorbed Serum 348, dose 0.75 cc. contained about 4 m.p.u.	(*c*)	17	Marked	Controlled	S		
		18	"	"	D_7	Sterile	Sterile
		19	Very marked	" 2 days	D_3	500	"
		20	" "	"	D_3	Sterile	"
		21	" "	" 4 days	D_6	Innumerable	Innumerable

Absorbed Serum 348, 0.2 cc.—1 m.p.u. + Serum 624, Type I, 0.3 cc.—300 m.p.u.§	(c)	22	Very marked	Controlled	S		
		23	Slight	" 2 days	D_7‡	Innumerable	Positive
		24	Very marked	"	D_{10}	Sterile	Sterile
		25	Moderate	"	S		
		26	Marked	"	D_9	Sterile	Sterile
Absorbed Serum 348, 0.5 cc.—2.5 m.p.u. + Serum 624, Type I, 0.3 cc.—300 m.p.u.	(c)	27	Slight	Controlled	S		
		28	Marked	Uncontrolled	D_3	Innumerable	Positive
		29	Very slight	Controlled	S		
		30	Slight	"	S		
		31	Very marked	"	D_{11}	Sterile	Sterile
Absorbed Serum 348, 1.0 cc.—5 m.p.u. + Serum 624, Type I, 0.3 cc.—300 m.p.u.	(c)	32	Slight	Controlled	S		
		33	Marked	"	S		
		34	Very marked	"	S		
		35	Marked	"	S		
		36	Very slight	"	S		

S = survived; D_4 = dead 4 days after infection. *M.p.u.* = mouse protective units.

* With a negative lesion, absent bacteremia, and entirely negative postmortem findings, the cause of death is not clear.

† With no gross dermal lesion, there were post mortem a hemorrhagic infiltration of both lungs and an acute fibrinous pericarditis.

‡ Hemorrhagic infiltration of both lungs; acute pleuritis and acute pericarditis.

§ See Tables II, III, and IV for effects of subeffective dose of Serum 624 when administered by itself.

Effect of the Non-Antibacterial Therapeutic Factor per Se

It was essential to determine the effect, if any, of a pneumococcus-absorbed serum, that is known to contain the non-antibacterial factor, when injected by itself without the addition of a certain subeffective dose of antibacterial serum. This experiment was expected to yield information regarding the rôle of the non-antibacterial factor as well as of the mouse protective antibody which is absorbable by the intact organism.

Two preparations were tested: (*a*) absorbed Preparation 4 which has been described previously, and (*b*) absorbed Serum 348, which was an unconcentrated monovalent Type I serum of at least 2000 m.p.u. per cc., and which after absorption with the homologous pneumococcus still contained about 5 m.p.u. per cc. The dose of absorbed Preparation 4 for each rabbit was 2 cc.; the dose of absorbed Serum 348, 0.75 cc. Three groups of rabbits were tested on different occasions; the results are shown in Table V.

It is necessary to recall that both Preparation 4, which is a concentrated (about 5 times) globulin preparation, and Serum 348 were so treated that the final absorbed preparations contained no *in vitro* demonstrable agglutinins, which involves a neutralization of the immune bodies not only by the SSS but by any other antigen that may be present on the intact organism. Whether or not the small amount of mouse protective antibody which is still demonstrable in these absorbed preparations is of an antibacterial or non-antibacterial nature cannot be definitely stated as yet, for it is not improbable that during the absorption of antibody an equilibrium may be reached wherein a certain small amount of antibody remains uncombined. Similarly it must be stated that the absorbed preparations probably do not contain the total quantity of non-antibacterial factor that is present in the serum since some of it may become non-specifically adsorbed during the precipitation of antibody on the bacteria.

The results presented in Table V show most interestingly, particularly in view of the fact that untreated rabbits do not survive with this dose of pneumococcus (see Table IV), that a certain number of rabbits is protected by the completely absorbed serum; eight of the sixteen rabbits treated with absorbed Preparation 4 and one of the five treated with absorbed Serum 348 were fully protected. A simul-

taneous titration of absorbed Serum 348 administered with a standard subeffective dose of Serum 624, indicates that approximately 0.5 to 1.0 cc. is efficient for rendering it effective. The difference in survival rate between the absorbed Preparation 4 and absorbed Serum 348 groups appears to be a matter of dosage more as regards the non-antibacterial factor than the antibacterial antibody, for the rabbits that died in the former group had a terminal septicemia, whereas two of the rabbits (Nos. 18 and 20) in the latter group died without any bacteremia but apparently by absorption of toxin from the marked focal lesions. Furthermore, it is important to note that in fourteen of the first twenty-one rabbits, the bacteremia was completely controlled. Whatever the nature of the residual mouse protective antibody, the amount, contained in the doses that were used, is relatively so very small that one is led to think that either only a very minute quantity of the antibacterial antibody is required for the control of bacteremia, or else that it plays a secondary rôle particularly when septicemia becomes very marked. In the last three groups of rabbits (Nos. 22 to 36), one observes how with a relatively standard mouse protective unit dose, the bacteremia is more readily controlled as the dose of the non-antibacterial factor is increased. With the larger dose of the non-antibacterial factor, as in absorbed Preparation 4, most of the rabbits showed very mild dermal lesions as compared not only with control, untreated rabbits but also with those which received an insufficient dose. When one contrasts with this the action of a standard subeffective dose that contains a relatively large amount of antibacterial antibody, in which case the dermal lesions are marked and death occurs frequently without bacteremia, the suggestion is very strong that the ultimate outcome of the infection depends to a great extent upon the non-antibacterial therapeutic factor. The effect of the non-antibacterial factor suggests that it opposes, in some way as yet unknown, the action of the products of infection which locally are responsible for a marked dermal lesion, and systemically are probably the cause of death.

DISCUSSION

The work reported in this communication is in line with an attempt to determine the mechanism of death and recovery in pneumococcus

infection. The rôle of the antibacterial antibodies, which are capable of combining with the various constituents of the bacterial protoplasm, is ultimately, as was pointed out by Bull, to enhance phagocytosis of the organisms by cells of the blood and tissues. With the thought that the mere multiplication of organisms as so many microscopic particles cannot logically be the cause of death, the view that phagocytosis is the chief factor which determines death or recovery appears to be unsatisfactory in many respects. The constituents which are isolated from virulent pneumococci *in vitro* and against which antibodies are demonstrable in the standard antipneumococcic sera, are non-toxic; yet when the intact, living organisms are introduced into the body of a susceptible animal, a most potent toxin is apparently elaborated. The question arises, therefore, whether antipneumococcic serum is effective only when through preventing the multiplication of the organisms it also prevents the formation of a lethal amount of toxin, or whether it possesses an action against this toxin as well.

Since in the method of mouse protection, which is at present used as the criterion for determining the potency of antipneumococcic sera, the serum is injected simultaneously with a relatively very small number of organisms into the same body cavity, the function which is primarily involved is that which brings about their phagocytosis, and prevents the multiplication of organisms to the extent that a lethal dose of toxin is not produced. For this reason this method was deemed unsuitable in the present study. The dermal pneumococcus lesion of the rabbit was chosen because it provided a focus where the organisms may be localized and from which, under conditions which are unsuitable for the systemic multiplication of the bacteria, sufficient toxin may be absorbed to cause death of the animal.

The first serum to be investigated was one prepared by the late Dr. E. J. Banzhaf, who immunized several horses not only by the intravenous injection of sedimented pneumococci (which is the routine practice), but also by the additional intramuscular injection of broth culture filtrates. Preliminary experiments showed that when certain similar doses, as regards mouse protective potency, of Banzhaf's serum and one prepared by the standard procedure, were tested on rabbits with the experimental dermal pneumococcus infection, the former was found to be fully protective and the latter practically in-

effective. It was thus apparent that the protective effect did not depend only on the number of mouse protective units and that some factor which was not determined by the mouse protection test, was present in one serum and absent in the other, or else present in both, but in different concentrations. The subsequent experiments were designed to determine whether this factor was of antibacterial or non-antibacterial nature. By absorbing an antipneumococcic serum with a heat-killed suspension of homologous pneumococci it is possible to remove almost all (about 99.5 per cent) of its mouse protective antibody. When this antibody was absorbed from the serum, prepared by immunization with organisms and broth culture filtrates, and the remaining supernatant liquid was administered with a subeffective dose (see Table II) of the original serum, it was found that this combined therapy proved fully effective. It appeared, therefore, that the difference between an effective and ineffective dose was not only the antibacterial antibody (*i.e.* that which is absorbed with the intact organisms) but also another factor which is apparently non-antibacterial. Furthermore, it was shown that a certain dose of such an absorbed preparation was by itself therapeutically effective in about half the treated rabbits. In the same experiment an analysis of the course of the bacteremia in the various rabbits indicated that either only a minute amount of the antibacterial antibody is required for the control of the bacteremia or else that it plays merely a secondary rôle only when the septicemia becomes very marked. These results again pointed to the fact that the ultimate outcome of the infection is dependent to a considerable extent upon the non-antibacterial therapeutic factor.

However, no definite causal relationship could be established between the additional injections of broth culture filtrates for immunization and the presence in the serum of the non-antibacterial factor, because (*a*) a concentrated preparation of broth culture filtrate failed to neutralize it, and (*b*) standard serum, prepared by the intravenous injection of sedimented organisms only, also contained this factor. It was further demonstrated that the non-antibacterial factor is not type-specific, and that it is probably species-specific. If this factor is of the nature of an antibody or an antitoxin, it must necessarily have its corresponding antigen. Such an antigen, by the very definition of the non-antibacterial factor, is not present in the intact or-

ganism; a concentrated broth culture filtrate did not contain it; and judging from the results obtained in these experiments, it is apparently also absent in the *in vitro* anaerobic autolysates of pneumococcus; however, it is not inconceivable that it may originate from the pneumococcus *in vivo*. From this point of view, it is interesting to consider the recently proposed conception of Curphey and Baruch (18). On purely theoretical grounds, they proposed the possibility that the immune bodies developed in the course of lobar pneumonia include antibodies which are formed as a result of the products of tissue cell destruction either *per se* or in combination with certain bacterial products. On the basis of this conception, they have prepared antipneumococcic serum by immunization with pleural exudates from pneumococcus-infected horses. In comparing (19) similar doses, as regards mouse protective units, of their serum with that of standard serum, using the dermal pneumococcus infection in rabbits, they found the former therapeutically more effective; however, since standard sera were shown to contain a non-antibacterial factor, and on account of the fact that the volume of the doses of the two sera was not taken into consideration, one cannot definitely assign the greater therapeutic effectiveness to the mode of immunization. The possibility, nevertheless, remains and deserves further investigation. It is also interesting to correlate the non-antibacterial therapeutic factor with Tillett's (20) observations on the production of immunity in rabbits against virulent strains by injections of R pneumococci; this immunity was not type-specific, and the serum of rabbits thus immunized, although it contained no demonstrable mouse protective antibody, was, nevertheless, capable of protecting normal rabbits against virulent Types I, II, and III pneumococci.

The fact, that antipneumococcic serum was shown to contain a therapeutically active non-antibacterial factor, may have an important bearing on the serum treatment of pneumococcus pneumonia in man, and suggests many new problems some of which have been investigated and will be reported in the next communication.

CONCLUSIONS

1. Antipneumococcic serum contains in addition to the antibodies against the various bacterial constituents, a non-antibacterial therapeutic factor.

2. The non-antibacterial factor has been separated from the antibacterial antibodies by absorption with homologous, heat-killed, virulent pneumococci.

3. The non-antibacterial factor is not type-specific; it is probably species-specific; it is not neutralized by concentrated pneumococcus broth culture filtrate.

4. The therapeutic effect of the non-antibacterial factor was demonstrated on the experimental, dermal pneumococcus infection of rabbits; this effect is demonstrable when it is administered *per se* or in conjunction with a certain subeffective dose of serum.

5. Evidence is presented for the assumption that the ultimate outcome of pneumococcus infection depends to a considerable extent upon this non-antibacterial factor.

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